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The Approximate Determination of Protein in Physiological Fluids

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Introduction.

Most of the methods proposed for determination of protein matter in animal fluids are based either on the principle of coagulating the protein by heat and directly weighing it as such, or on the principle of precipitating the substance with special reagents and subjecting the precipitated protein matter to the Kjeldahl method of nitrogen determination. Among the special reagents are to be mentioned magnesium sulphate,[1] ammonium sulphate[2], zinc sulphate,[3] potassium aluminium sulphate,[4] copper sulphate,[5] suspended cupric hydrate,[6] tannic acid[7], and phosphotungstic acid.[8]

The quantitative methods based on measuring the volume of precipitates formed in a solution have recently attracted attention of chemists. Such are, for example, the micro-volumetric analysis of potassium by H. J. Hamburger,[9] and the similar method of determination of platinum

1 Hoppe-Seyler and Tolmatsheff, Hoppe-Seylers Med. Chem. Unters., H. 2.

2 Hofmeister u. Pohl, Arch. f. Exp. Path. u. Pharm., **20**; Devoto, Zeits. f. Physiol. Chem., **15**, 474 (1891); Hammarsten, Pflügers Arch., **17**.

3 Morawitz u. Dietschy, Arch. f. exp. Path. u. Pharm., **54**.

4 Schlossmann, Zeits. f. Physiol. Chem., **22**.

5 Ritthausen, J. f. Prakt. Chem. (N.F.) **15**.

6 J. Munk, Virch. Arch., **134**.

7 Sebelien, Zeits. f. Physiol. Chem., **13**, (1889).

8 G. Simon, Zeits. f. Physiol. Chem., **33** (1901).

9 Hamburger, Recueil d. Trav. Chim., **35**. 225–253 (1916).

in presence of gold and silver by M. Van Breukeleveen.[1] A similar precipitato-volumetric method for protein matter has long been known under the name of the Esbach's method,[2] and widely used by clinicians for an approximate determination of albumin in urine. It consists in adding to a definite volume of acid-reacting urine or urine acidified with acetic acid a definite volume of the reagent containing 2 per cent of citric acid and one per cent of picric acid, and reading the volume of the precipitate formed after 24 hours' standing in the room temperature. There have various modifications been proposed for this method.[3]

A much simpler, but far more reliable, approximate estimation of protein can be made by the Roberts-Stolnikoff-Brandberg modiffcation of the Heller's test.[4] It is based on the observation that the water or urine containing one part of protein in 30,000 parts, i.e. 0.0033% of protein, gives the Heller's test of white disk or ring in 2½ to 3 minutes, when carefully superposed on a layer of concentrated nitric acid in a test tube.

The purpose of the present investigation is to find some more time-saving methods or modifications of the existing methods for approximate estimation of small quantities of protein in animal fluids, esp. in urine. It is believed that this object has been partially realised in the three different ways to be sketched in this paper.

I. Acidimetric Estimation of Protein in Solution.

Picric acid is an excellent precipitant for protein in solution,[5] although in such a liquid as urine it also precipitates alkaloids, cre-

1 Van Breukeleveen, Recueil d. Trav. Chim., **36**, 285 (1917).

2 G. Esbach, Bulletin de Therapie, Janv. **1874**; Gazette Med. de Paris, **5**, 61 (1874). H. Veale, Brit. Med. J., **1**, 898 (1884). H. E. Jones, Glasgow Med. J., **85**, 31–35 (1916), Chem. Abst., **11**, 612 (1917). Guttmann, Berl. Klin. Woch., **23**, 117 (1886). Hammarsten, Lehrb. d. Physiol. Cem. 747 (1914). Jaksch, Klin. Diagnostik, 393–395 (1901). Czapek, Prager Med. Woch. **13**, 128 (1888). Sokolow, Maly's Jahresber., **17**, 223 (1888) Referat; Geissler, Berl. Klin. Woch., **26**, 111 (1889).

3 Neubauer-Huppert, Analyse des Harns, Bd. **2**, 1142–1148 (1913).

4 J. F. Heller, Arch. f. Physiol. u. Path. Chem. u. Mikroscopie, **5**, 161 (1852). Roberts, The Lancet, I, 313 (1876). Stolnikow, Maly's Jahresb. f. Thier-Chemie, **6**, 148, Referat, (1877). J. Brandberg, Maly's Jahresb. f. Thier-Chemie, **10**, 265, Referat, (1881). Mittelbach, Prager Med. Woch. **23**, 381, (1898). Hammarsten, Maly's Jahresb., **10**, 265, Referat (1881), **13**, 217, Referat (1884). Jaksch, Klin. Diagnostik, 391–393 (1901).

5 G. Johnson, On the Various Modes of Testing for Albumen and Sugar, Smith Elder & Co., London, p. 6, (1884).

atinine, uric acid, etc.[1] For the precipitation purpose, previous authors have always used the picric acid in presence of some other organic acids, such as citric or tartaric acids, as exemplified in the well-known Esbach's method mentioned above. The present author has, however, found that dilute hydrochloric acid is a much more convenient adjunct for picric acid for this precipitation purpose, and that the mixture of these two acids can be very easily titrated with phenolphthalein as indicator. The standard solution of picric acid is also very easily made, for the saturated solution is at the ordinary temperature quite nearly of the twentieth-normal strength. The molecular weight of picric acid is 229.15 while its saturated solution generally contains about 1.2% of the acid. The optimum acidity of hydrochloric acid in the liquid from which the protein is to be precipitated is found to be the fifteenth to twentieth normal. The following experiments show that there is a definite quantitative relation between the albumin in solution and the picric acid required to completely precipitate it.

Experiment I. Four parallel experiments were started, in each of which 10 c.c. of a solution of dry serum albumin in 0.7% sodium chloride solution, containing 242 mg. of the albumin as determined by the Kjeldahl method, were mixed with 15 c.c. of nearly N/5 hydrochloric acid, 12.5 c.c. of 0.7% sodium chloride solution, and 7.5 c.c. of 1.2% picric acid solution, so as to make a volume of 45 c.c. in total. By separate titrations, it was found that the acidity, with phenolphthalein as indicator, of the 10 c.c. of the albumin solution was equal to 0.4 c.c. of the N/10 alkali solution and that of 7.5 c.c. of 1.2% picric acid solution was equal to 4.1 c.c. of the same alkali. Thus the initial total acidity of the 45 c.c. of the mixed liquid was equal to 34.15 c.c. of the N/10 solution. All the four mixtures were now placed in a water bath at the temperature of 35–37°C., and kept there with occasional shaking for one hour, in order to make the precipitate more compact and more easily filtrable. After removing from the bath, each was filtered through a small dry filter paper, and 20 c.c. of the filtrate were titrated with N/10 sodium hydroxide solution with the following result:

1 M. Jaffé, Zeits. f. Physiol. Chem., **10**, 399 (1886).

	Acidity in c.c. of N/10 NaOH Solution.				
	I	II	III	IV	Average
Total of initial acidity	34·15	34·15	34·15	34·15	34·15
Acidity of 20 c.c. filtrate	13·55	13·53	13·57	13·60	13·57
Acidity calculated for 45 c.c. filtrate					30·53
Acidity of picric acid neutralised by 242 mg. of albumin					3·62

The filtrate and the washings showed no albumin reaction with the Heller's test. It is calculated from the above result that 242 mg. of serum albumin are completely precipitated by 3.62 c.c. of N/10 picric acid solution equal to 7.24 c.c. of the 1.146% reagent. 1 c.c. of the N/20 picric acid solution will thus precipitate 33.4 mg. of the serum albumin.

Experiment II. In this series of four parallel experiments, 10 c.c. of the serum albumin solution containing 242 mg. of the albumin were carefully neutralized with the N/10 sodium hydroxide solution and then mixed with 15 c.c. of nearly N/5 hydrochloric acid and with the varying volumes of 1.2% picric acid and of 0.7% sodium chloride solutions as shown below. The total volume of the mixtures was always 45 c.c. (15 c.c. of the hydrochloric acid solution were equal to 29.65 c.c. of the N/10 alkali solution, while 10 c.c. of the picric acid solution were equal to 5.55 c.c. of the same alkali.)

	I	II	III	IV
Serum albumin solution (c.c.)	10·0	10·0	10·0	10·0
Nearly N/5 HCl (c.c.)	15·0	15·0	15·0	15·0
Saturated picric acid solution	7·5	10·0	15·0	20·0
7 per mille sodium chloride	12·5	10·0	5·0	
Total volume in c.c.	45·0	45·0	45·0	45·0

All the four mixtures were now immersed in the water bath at the temperature of 36–37°C., with occasional shaking, for one hour. They were then filtered through dry filter paper, and 35 c.c. of the filtrate were taken from each sample and titrated with the N/10 alkali solution.

	Acidity in c.c. of N/10 NaOH solution.				
	I	II	III	IV	Average
Total of initial acidity	33·82	35·20	37·98	40·75	
Acidity of 35 c.c. filtrate	23·15	24·30	26·10	28·35	
Acidity computed for 45 c.c.	29·76	31·24	33·56	36·45	
Acidity of picric acid neutralized by 242 mg. albumin	4·06	3·96	4·42	4·30	4·15

It is calculated from the above result that 242 mg. of serum albumin are completely precipitated by 4.15 c.c. of the N/10 solution of picric acid equal to 8.3 c.c. of the 1.146% reagent. 1 c.c. of the N/20 picric acid solution will thus precipitate 29.2 mg. of the serum albumin.

Experiment III. 10 c.c. of the serum solution containing 242 mg. of the albumin were diluted to 40 c.c. with 0.7% sodium chloride solution and then mixed with 12 c.c. of nearly N/5 hydrochloric acid. The mixture was now divided into four equal parts of 13 c.c. each. To each of the first three parts, there were added 2 c.c. of a saturated picric acid solution equal in acidity to 1.11 c.c. of the N/10 solution. The first part was then filtered at once, while the second and the third were immersed in the water bath at the temperature of 35–36° with occasional shaking for one hour, when they were filtered. 10 c.c. were taken from each filtrate and titrated, with phenolphthalein as indicator, against N/10 NaOH solution. (In the fourth part of the original mixture, the acidity was determined and found to be exactly equal to 60 c.c. of the N/10 alkali solution). It is seen from the following tabulated result that 242 mg. of serum albumin are completely precipitated by 4.12 c.c. of the N/10 picric acid solution. 1 c.c. of the N/20 acid wlll thus precipitate 29.4 mg. of serum albumin.

	Acidity in c.c. of N/10 NaOH solution			
	I	II	III	Average
Total of initial acidity	7·11	7·11	7·11	7·11
Acidity of 10 c.c. filtrate	4·05	4·05	4·07	
Acidity computed for 15 c.c.	6·08	6·08	6·10	6·08
Acidity of picric acid neutralized by 60·5 mg. albumin				1·03
Acidity of picric acid computed for 242 mg. albumin				4·12

In applying the principle illustrated in the above experiments to the estimation of albumin in urine, many factors are to be taken into consideration. As phenolphthalein is used as indicator for titrating the excess of picric acid, it is very important to exclude the influence of carbon dioxide either from the air or from the experimenter's breath. If this caution is neglected a serious error might creep in to vitiate the whole operation. As the weight of the albumin precipitated by 1 c.c. of N/10 picric acid makes so large a figure as 58.4–66.8 mg., the reading of the burette must be done with an utmost care so as to minimize the error arising from this source. It is, therefore, advisable to choose a burette of as narrow a caliber as possible. As the liquid in which albumin is to be estimated must be of nearly N/20 strength in hydrochloric acid, and as this acidity is to be titrated with N/10 NaOH solution both before and after adding the picric acid solution, it will be advisable to take, as a rule, 30 c.c. of the test urine and add to it 10 c.c. of N/5 HCl before adding the required amount of picric acid. Finally, as the normal urine contains creatinine and uric acid and it may contain, in pathological conditions, such substances as quinine, antipyrine, thalline, and other alkaloids, which are precipitated by picric acid, there must always be performed a control experiment to ascertain to what an extent these substances partake in the consumption of the acid. The following preliminary studies of the change of acidity of the urine under different treatments may be appropriately recorded here.

Experiment IV. The urine taken was of specific gravity 1.025; its albumin content was 120 mg. per 1000 c.c. according to my modification of Roberts-Stolnikow method; it was weakly alkaline toward

litmus, but toward phenolphthalein it had an acidity equivalent to 8/1000—N sodium hydroxide solution. Four parallel experiments were performed in each of which 10 c.c. of the urine were mixed with 3 c.c. N/5 HCl and 2 c.c. N/20 picrid acid solution. The first of these mixtures was immediately filtered and titrated with 10 c.c. of the filtrate against N/10 sodium hydroxide solution. The remaining three were allowed to stand 19 hours and then filtered and titrated with 10 c.c. of the filtrate against the same N/10 alkali solution.

	Acidity in c.c. of N/10 NaOH solution				
	I	II	III	IV	Average
Initial acidity of 10 c.c. urine	0·80	0·80	0·80	0·80	
„ „ „ 3 c.c. HCl	6·00	6·00	6·00	6·00	
„ „ „ 2 c.c. picric acid	1·00	1·00	1·00	1·00	
Total of initial acidity	7·80	7·80	7·80	7·80	7·80
Acidity of 10 c.c. filtrate	4·60	4·35	4·50	4·55	
Acidity computed for 15 c.c.	6·90	6·52	6·75	6·82	6·75
Acidity destroyed in 10 c.c urine					1·05

With the above urine, an estimation of added albumin was performed in two parallel experiments:— 8 c.c. of the urine, 2 c.c. of N/20 picric acid, 2 c.c. of albumin solution containing 48.3 mg. of the substance, and 3 c.c. of N/5 hydrochloric acid were mixed together. The total of acidity of the four components was 7.72 c.c. N/10 solution and the total volume was 15 c.c. The mixtures were allowed to stand 20 hours and then filtered, and the acidity was measured in 10 c.c. of the filtrate with N/10 sodium hydroxide solution.

	Acidity in c.c. N/10 NaOH solution.		
	I	II	Average
Total of acidity of components	7·72	7·72	7·72
Acidity of 10 c.c. filtrate	4·20	4·15	
Acidity computed for 15 c.c.	6·30	6·23	6·27
Acidity destroyed by urine+albumin			1·45

By subtracting from the figure 1.45 thus obtained the figure 0.84 for the acidity destroyed by 8 c.c. of the urine alone, it is found that 48.3 mg. of albumin have precipitated 0.61 c.c. of the N/10 picric acid.

In another word, 1 c c. of the saturated picric acid solution (N/20) has precipitated 39.6 mg. albumin.

Experiment V. A fresh urine of sp. gr. 1.025 had an acidity equal to 15/1000−N solution. When 10 c.c. of this urine were mixed with 3 c.c. of N/5 hydrochloric acid and filtered and titrated, the acidity of the urine was found to have decreased to 12/1000−N. In three more parallel experiments, 10 c.c. of the urine, 3 c.c. of N/5 hydrochloric acid, and 2 c.c. of N/20 picric acid were mixed together and kept for one hour in the water bath at the temperature of 37°C. Each mixture was then filtered and 10 c.c. of the filtrate were titrated as usual.

	Acidity in c.c, of N/10 NaOH solution.			
	I	II	III	Average
Total of acidity of components	8·45	8·45	8·45	8·45
Acidity of 10 c.c. filtrate	5·35	5·35	5·40	
Acidity computed for 15 c.c.	8·03	8·03	8·10	8·05
Acidity neutralized by 10 c.c. urine				0·40

In two more parallel experiments, 8 c.c. of the above urine, 3 c.c. of N/5 hydrochloric acid, 2 c.c. of serum albumin solution containing 48.2 mg. of the substance, and 2 c.c. of N/20 picric acid solution were mixed together and kept for one hour in the water bath at the temperature of 37°C. The mixtures were then filtered and the acidity was titrated in 10 c.c. of the filtrate as usual.

	Acidity in c.c. of N/10 NaOH solution.		
	I	II	Average
Total of acidity of components	8·24	8·24	8·24
Acidity of 10 c.c. filtrate	4·75	4·70	
Acidity computed for 15 c.c.	7·13	7·05	7·09
Acidity destroyed by urine+albumin			1·15

By subtracting from the figure 1.15 thus obtained the figure 0.32 for the acidity neutralized by 8 c.c. of the urine alone, it is found that 48.2 mg. of albumin have precipitated 0.83 c.c. of the N/10 picric acid. In another word, 1 c.c. of the saturated picric acid solution of N/20 strength has precipitated 29.2 mg. serum albumin.

A study of the practical application of this principle to the estimation of albumin in pathological urines is now going on in my laboratory.

II. PRECIPITATO-VOLUMETRIC METHOD OF DETERMINING PROTEIN IN SOLUTION.

The Esbach's method in ordinary use among clinicians for determining protein in urine requires, as was stated above, a solution containing one per cent of picric acid and two per cent of citric acid, besides a specially constructed "albuminimeter." It also requires 24 hours' standing of the reaction mixture at the room temperature.

According to the precipitato-volumetric method to be described here, the reagents used are 1.2% picric acid solution and the standard normal solution of hydrochloric acid in the ordinary urine. The apparatus used are a set of three ordinary measuring cylinders or conical graduates of 20 c.c. capacity graduated to half a cubic centimeter, and a water-bath with a flat bottom to receive these graduates. The time required to elapse before reading the volume of the precipitate is three or four hours. Thus there is secured by this new method an economy both in time of operation and in costs of reagents and apparatus. The procedure in practice in my laboratory is as follows :

Take the graduated cylinders or cones, each of 20 c.c. capacity, and make three parallel experiments by filling each with 10 c.c. of the test urine, 1 c.c. of the standard normal hydrochloric acid solution in a healthy urine, 2 c.c. of 1.2% picric acid solution, and 2 c.c. of the healthy urine. The total volume is now 15 c.c. The graduates are placed in the water-bath with a flat bottom, and kept for one hour at the temperature of 35–37°C. with an occasional stirring with a glass rod. They are now removed from the bath and allowed to stand for 3 hours more, and then the volume of the precipitate is read. The number of c.c. of the precipitate gives, without any farther computation, the number of grams of albumin per liter of the urine.

An important factor for the success of this method is the concentration of the reaction fluid from which the protein is to be precipitated. The test urine must be of a sp. gr. near 1.010. If it is higher it must be diluted before the use, or otherwise a correction must be made on the readings of the volume of the precipitate. In diluting a urine of high specific gravity to get that of sp. gr. 1.010, the degree

of the necessary dilution is easily known by looking at the number represented by the second and third decimal figures. For example, if the test urine has a sp. gr. of 1.035, diluting it to 3.5 times its volume will bring it to the sp. gr. of 1.010. Again if the test urine has a sp. gr. of 1.028, diluting it to 2.8 times its volume will bring it down to the sp. gr. 1.010. This follows from the observations underlying the Häser's and Long's coefficients[1]. Another important condition for the success of this method is the temperature of the water-bath which must be kept rigorously at 35–37°C.

In case one is content with making only one single experiment, it is proposed that he use a tube similar to the Esbach's albuminimeter but graduated in cubic centimeters. Best suited as the water bath for the single tube experiment is a wide mouthed bottle, 12–13 cm. high and of about 150 c.c. capacity, in whose cork stopper there is a large hole bored just to admit the albuminimeter tube. This bottle is filled with warm water of temperature of 38–40° and at once well wrapped with a piece of flannel. The albuminimeter is now inserted and allowed to stand, with occasional removing and shaking, for one hour. In a warm season it is not difficult thus to keep the required tempereture of the bath at 35–37°C. for a long time.

In the following experiments I used a set of ten tubes of about 25 c.c. capacity, of the bore of an ordinary burette, and with ground glass stoppers, and graduated to one-tenths of a cubic centimeter. A suitable water bath was improvised from an ordinary Soxhlet apparatus for disinfecting milk by modifying the inner rack so as to hold in erect position those ten tubes and a thermometer. The desired temperature of 35–37°C. is reached by only a few minutes' heating.

Experiment VI. The fresh urine used in this experiment was passed by a man, N. R., aged 47, suffering from an incipient chronic nephritis. This particular sample contained no albumin detectable with the Heller's test. It had the sp. gr. of 1.010 and contained 0.71% sodium chloride. 10 c.c. of it titrated with phenolphthalein corresponded to 0.7 c.c. of N/10 NaOH solution. Some dried serum albumin was finely powdered and rubbed with the urine and then filtered. In a portion of it the albumin was determined by combination of the heat coagulation and Kjeldahl digestion and was found to amount to 1.44%.

[1] Long, Biochem. Zentralbl, **1**, 515 & 703. Hammarsten, Lehrb. d. Physiol. Chem., 726 (1914).

This albuminated urine was then diluted with the original album n-free urine to five different strengths varying from 1 to 5 gr. albumin per 1000 c.c. To 10 c.c. of the albuminated urine were added 1 c.c. of 1-N solution of hydrochloric acid in albumin-free urine, 2 c.c. of 1.2% picric acid, and 2 c.c. of the albumin-free urine, so as to make the total volume of 15 c.c. and resulting total acidity of N/15. The following results were obtained by proceeding as directed above:

No.	Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing			
		3 hours	4 hours	5 hours	24 hours
1	1	1·20	1·15	1·15	1·15
2	1	1·10	1·10	1·10	1·10
3	1	1·35	1·35	1·35	1·30
4	1	1·15	1·20	1·20	1·15
5	1	1·50	1·25	1·25	1·20
6	1	1·50	1·30	1·20	1·20
average	1	1·30	1·20	1·20	1·20
7	2	2·40	2·35	2·35	2·20
8	2	2·40	2·30	2·30	2·15
9	2	2·25	2·20	2·15	2·10
10	2	1·95	1·90	1·90	1·80
11	2	2·50	2·15	2·00	1·90
12	2	2·65	2·30	2·10	2·10
average	2	2·35	2·20	2·10	2·10
13	3	4·1	3·8	3·7	3·35
14	3	2·5	2·4	2·4	2·25
15	3	2·8	2·7	2·7	2·60
16	3	2·6	2·5	2·5	2·40
17	3	3·5	3·2	2·8	2·50
18	3	3·5	3·1	3·0	2·75
average	3	3·2	2·9	2·8	2·6
19	4	3·3	3·1	3·0	2·9
20	4	4·2	3·7	3·6	3·3
21	4	4·1	3·8	3·7	3·4
22	4	4·9	4·6	4·5	4·0
23	4	4·5	4·0	3·6	3·3
24	4	5·0	4·6	4·2	3·8
average	4	4·3	4·0	3·8	3·4

Test urine	Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing			
		3 hours	4 hours	5 hours	24 hours
25	5	7·1	5·6	5·0	4·1
26	5	6·0	4·8	4·5	3·8
27	5	5·7	4·9	4·7	4·0
28	5	6·3	5·6	5·2	4·2
29	5	5·5	5·0	4·5	3·8
30	5	5·7	5·1	4·7	4·1
average	5	6.1	5·2	4·4	4·0

From the above table it is seen that the number of cubic centimeters of the precipitate after standing 4 hours represents quite closely the number of grams of the albumin in 1000 c.c. of the test urine.

Experiment VII. The influence exerted by the specific gravity of the test urine upon the volume of the precipitate formed may be seen by comparing the foregoing data with those to be given below. If the specific gravity becomes higher, the volume of the precipitate becomes greater and its rate of increase more irregular.

The test urine K.N. was prepared by mixing the strongly albuminated urine mentioned under Exp. VI. with a non-albuminous urine of sp. gr. 1.025, of the sodium chloride content of 0.8% and of the acidity of N/25 with phenolphthalein. This latter urine was obtained from a 9-year old girl who had recently recovered from acute nephritis and was suffering at the moment from the orthotic albuminurea.

The test urine T.K. was prepared in the similar manner from a non-albuminous urine passed by a man, 25 years old, in normal health. The latter urine had sp. gr. 1.026, sodium chloride content 2%, and the acidity of N/22 with phenolphthalein.

The test urine E.N. was prepared by dissolving dried serum albumin in a non-albuminous urine, sp. gr. 1.025, passed by a 10-year old girl in perfect health. A weighed amount of the dried albumin, the protein content of which was known by the Kjeldahl method, was rubbed with a small quantity of the urine with an addition of a little of N/10 sodium hydroxide solution, then neutralized with N/10 sulphuric acid, and finally diluted with the same urine to the desired albumin content.

The test urine N.K. was prepared in a similar manner from a non-albuminous urine of a pregnant woman in perfect health. The latter urine had sp. gr. 1.023, sodium chloride content 1.1% and the acidity N/40 toward phenolphthalein. It also had a sediment showing many lymph corpuscles with some crystals of uric acid and of calcium oxalate; the sediment was of course filtered off before the urine was taken for the experiment.

Test urine	Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing			
		3 hours	4 hours	5 hours	24 hours
E.N.	0·3	0·7	0·6	0·6	0·6
N.K.	0·3	0·7	0·7	0·7	0·7
Average	0·3	0·7	0·7	0·6	0·6
T.K.	0·7	1·4	1·4	1·3	1·3
E.N.	0·7	1·0	0·9	0·9	0·8
N.K.	0·7	1·3	1·2	1·1	1·1
Average	0·7	1·2	1·1	1·1	1·1
K.N.	1·0	1·6	1·5	1·5	1·3
T.K.	1·0	1·0	1·0	0·9	0·9
Average	1·0	1·3	1·3	1·2	1·1
E.N.	1·5	2.3	2·0	1·9	1·6
N.K.	1·5	2·2	2.0	1·8	1·7
T.K.	1·5	1·8	1·7	1·7	1·5
Average	1·5	2·1	1·9	1·8	1·6
E.N	2·0	3·0	2·5	2·4	1·9
N.K.	2·0	3·1	2·8	2·6	2.2
K.N.	2·0	2·7	2·6	2·5	2·3
T.K.	2·0	2·7	2·5	2·4	2·2
Average	2·0	2·9	2·6	2·5	2·2
K.N.	3·0	4·7	4·2	4·0	3·5
T.K.	3·0	4·2	3·8	3·6	3·1
E.N.	3·0	5·6	4·2	3·7	2·3
N.K.	3·0	3·6	3·3	3·0	2·5
Average	3·0	4·5	3·9	3·6	2·9

Test urine	Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing			
		3 hours	4 hours	5 hours	24 hours
E.N.	4·0	8·5	6·2	6·0	3·5
N.K.	4·0	6·2	5·6	5·1	3·8
K.N.	4·0	5·7	5·2	5·0	4·3
T.K.	4·0	5·6	4·9	4·5	3·7
Average	4·0	6·5	5·5	5·2	3·8

The following empirical rule may be deduced from the above table: "When a urine of sp. gr. 1.025 is treated, without a previous dilution, according to the direction given above, a correction should be made upon the readings for the 4th hour so that the readings below 1.0 are multiplied with factor 0.5 and those between 1.3 and 6.0 are multiplied with factor 0.8 The figures thus obtained will quite closely express the number of grams of the albumin in 1000 c.c. of the urine."

Experiment VIII. The fact that a protein content higher than 4 gr. per 1000 c.c. is apt to lead to a more or less irregular value is seen from the data to be given forthwith. The procedure here proposed can, therefore, be safely applied only on those urines that contain less than 5 per mille of protein, and the urines of higher protein contents must be first diluted with a non-albuminous urine so as to get a content of from two to five per mille.

Test urin	Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing for			
		3 hrs.	4 hrs	5 hrs.	24 hrs.
N.R.	5	6·3	5·8	5·3	4·6
K.N.	5	6·7	6·1	6·0	5·0
T.K.	5	10·2	8·2	7·1	4·6
N.R.	6	7·6	6·9	6·3	5·5
K.N.	6	7·4	7·0	6·7	5·6
N.R.	7	9·0	8·0	7·2	6·1
K.N.	7	9·0	8·2	7·8	6·2
K.N.	8	9·2	8·4	8·0	6·3

It must be noted here that the picric acid taken in this procedure —2 c.c. of the 1.2% solution—is just sufficient to completely combine about 60 mg. of albumin. Therefore it is interesting to see in the above table the fact that 60, 70, and 80 mgs. of albumin in 10 c.c. urine all give the same volume of precipitate after standing 24 hours.

Experiment IX. The volume of the precipitate is decidedly influenced by the concentration of picric acid as is seen from the figures here to follow. In this experiment, the only deviation from the general procedure consisted in using 3 c.c. of 1.2% picric acid instead of 2 c.c. and consequently omitting the addition of 2 c.c. of a non-albuminated urine.

Protein gr. per 1000 c.c.	Volume (c.c.) of precipitate after standing for				
	2 hrs.	3 hrs.	4 hrs.	24 hrs.	94 hrs.
6	4·0	3·8	3·2	3·0	2·9
7	5·2	4·8	4·5	4·3	4.0
8	5·2	4·8	4·5	4·1	4 0
9	5·1	4·9	4·3	4·1	4·0
10	5·3	4·8	4·3	4·0	3·9

The most interesting point here noted is the fact that between 7 and 10 grams albumin per 1000 c.c., the volume of the precipitate remains constant inspite of the increase of its absolute weight. That it is not due to any insufficient precipitation of the protein is shown by the Heller's test made on the supernatant liquid which gives now no ring characteristic of protein. It may be also recalled that by experiment given above under "Acidimetric Estimation of Protein in Solution," it was shown that about 30 mgs. of protein are completely precipitated by 1 c.c. of 1.2% picric acid solution under the condition of the present procedure. It may also be noted in this connection that Esbach assumes in his gravimetric estimation of protein[1] that 20 parts of picric acid combines with 80 parts of protein.

Experiment X. The complete precipitation of protein by picric acid takes place only in an acid medium and the different kinds of acids used have different influences on the volume of the precipitate. This point is shown by the experiments to follow.

1 See Neubauer-Huppert, Analyse d. Harns. II, 1142 (1913).

1. Each of five test tubes was filled with 10 c.c. of urine containing 29 mgs. of serum albumin. 1 c.c. of 1.2% picric acid did not cause any precipitation. Now five different acids were taken in equivalent quantities and added separately to the five mixtures. Their quantities were so calculated as to form N/35 solution. The precipitation took place at once and the tubes were then allowed to stand at the room temperature ranging from 15° to 20°C.

Acid added mgs.	Serum-Albumin mgs.	Volume (c.c.) of precipitate after standing			
		19 hrs.	22 hrs.	24 hrs.	48 hrs.
HCl 10	29	3·5	3·3	3·2	3·0
Citric 20	29	4·5	4·0	2·7	2·7
Tartaric 21	29	4·0	3·7	3·5	3·1
H_2SO_4 15	29	7·0	7·5	6·1	3·5
Acetic 18	29	7·0	6·5	6·1	3·5

2. Each of five test tubes was filled with 12 c.c. of urine containing 29 mgs. serum albumin. 0.5 c.c. of one-Normal solution of different acids were added together with 1 c.c. of 1.2% picric acid so as to make the resulting acidity of N/27. The tubes were allowed to stand at the room temperature ranging from 15° to 20°C.

1-N acid added c.c.	Serum-albumin mgs.	Volume (c.c.) of precipitate after standing				
		14 hrs.	17 hrs.	22 hrs.	40 hrs.	96 hrs.
HCl 0·5	29	5·5	4·8	4·0	3·1	2·6
HNO_3 0·5	29	5·2	4·5	3·7	3·2	2·8
Citric 0.5	29	7·7	6·9	5·6	3·5	2·6
Tartaric 0·5	29	7·2	6·4	5·0	3·5	5·9
Acetic 0·5	29	9·0	8·4	7·2	4·6	2·6

From the above figures it is easily seen that hydrochloric acid is the best acid suited for use in the present procedure.

Experiment XI. The acid strength has an influence upon the volume of the precipitate formed. One Normal and one-half-Normal solutions of hydrochloric acid in a non-albuminous urine were first prepared and different quantities were then mixed with an albuminated urine to get mixtures of varying resulting acidity. 2 c.c. of 1.2%

picric acid were then added to complete the total volume of 15 c.c. The room temperature ranged from 18° to 22°C.

Acidity of HCl	Serum-albumin mgs.	Volume (c.c.) of precipitate after standing					
		2 hrs.	3 hrs.	4 hrs.	17 hrs.	20 hrs.	70 hrs.
N/2	29	10·8	9·2	8·1	3·7		
N/3	29	9·5	7·7	6·6	3·4		
N/4	29	7 3	5·5	4·6	3·3		
N/5	29	5·7	4·2	3·7	3·0		
N/6	29	5·0	4·1	3·7	3·2		
N/8	29	4·4	3·6	3·3	2·8		
N/10	29	3·9	3·3	3·3	2·9		
N/12	29	3·6	3·3	3·2	3·0		
N/16	29	3·6	3·3	3·2	3·0		
N/20	29	3·5	3·3	3·1	2·8		
N/2	50	13·1	11·9	10·9		4·6	3·4
N/3	50	12·3	11·3	10·2		4·9	3·6
N/4	50	11.1	9·4	8·0		4·7	3·8
N/5	50	9·0	7·0	5·8		4·5	3·8
N/6	50	10·3	8·5	7·1		4·5	3·6
N/8	50	9·9	8·1	6·7		4·5	3·6
N/10	50	8·1	6·3	5·6		4·6	3·9
N/12	50	8·6	6·8	6 0		4·8	3·4
X/16	50	7·0	5·9	5·5		4·8	4·2
N/20	50	7·9	6·1	5·5		4·6	4·0

From the above figures it is seen that the acidity between N/10 and N/20 is the most suited for the present procedure. I have adopted the twentieth Normal strength (N/20) as the final acidity to be given to the reaction mixture.

III. SIMPLIFICATION OF THE ROBERTS-STOLNIKOW PROCEDURE OF ESTIMATING PROTEIN IN SOLUTION.

The procedure of Roberts and Stolnikow improved by Brandberg, Mittelbach, and O. Meyer,[1] as roughly sketched in the Introduction,

[1] See Neubauer-Huppert, Analyse d. Harns, II, 1137–1142 (1913).

can be further simplified and brought to the use not only of the busy clinicians but of every house-wife and sick-nurse. The great advantage of the wider popular use of this important diagnostic means will be at once recognized by the family physicians and will no doubt lead to an early detection of the incipience of many serious disturbances of nutrition and metabolism.

The procedure that I propose for this extended popular use is founded on the observation that, when a fluid containing protein in solution is subjected to the Heller's test, there is a definite relation between the quantity of protein and the time elapsing before the appearing of the distinct white ring. This relation is conveniently tabulated as below:

Protein mgs. per 100 c.c.	Time for distinct appearing of white ring
10	While adding HNO_3
8	Immediately on adding
6	½ minute after adding
5	1 „ „ „
4	1½ „ „ „
3	2½–3 „ „ „

The requirements for my procedure are:

1. Strong nitric acid of sp. gr. 1.25–1.4, that is to say of 40–60%.

2. Two conical measuring glasses of 10 c.c. capacity. The glass must be as nearly colorless as possible and free from any spots or roughness of surface, especially below the line for 2 c.c.

3. The glass tubes, about 20 cm. long and 6 mm. wide, provided with a small stout rubber bulb on the one end and drawn to a narrow point on the other. They are to serve as pipets of about 2 c.c. capacity, and have file marks at 1 c.c. and 2 c.c. points.

4. A slip of a thick paper board, 10 cm. long and 2 cm. broad. One face of this slip is smeared black to serve as the back ground for observing the white ring (or disk) of protein. On the other face is printed the abbreviated table showing in one glance the quantities of protein corresponding to the time observed.

Procedure. Take the test urine by means of the pipet directly from the urinal and fill the two measuring glasses each to 1 c.c. mark. Take with the other pipet about 0.7 c.c. of the nitric acid in such a

manner that there is no air bubble left at the tip. Reach with this pipet to the bottom of one of the measuring glasses and carefully squeeze out the acid so as to make the layer of the acid slowly push upward the layer of the urine already in the glass. When the acid is nearly all squeezed out, raise the end of the pipet just slightly above the contact surface of the two liquids and cautiously squeeze the remainder of the acid out and then quickly remove the pipet. Bubbling the air through the nitric acid is absolutely to be avoided, while bubbling it through the urine layer does not disturb the smoothness of the contact surface. Now watch the time elapsing after completing the above treatment and before the appearing of any distinct white disk of the precipitated protein. The proper way of making the observation is to face the window holding with the left hand the conical measuring glass before the eye and slowly moving with the right hand the narrow slip of black paper up and down on the other side of the contact line. The glass is kept at such a position that the eye looks obliquely upward at the contact line of the two liquids.

If, observed in the indicated way, the white disk or ring does not appear in three minutes, the test urine contains less than three milligrams in 100 c.c. Here it may be noted that according to K. Mörner[1] protein appears regularly as a normal constituent of urine in quantities varying from 2.2 to 7.8 mg. in 100 c.c. Von Leube[2] had previously shown that the urine of 4% of the healthy persons examined by him contained protein constantly, and that 16% of the healthy persons showed protein in their urine after a strained muscular exertion. This remarkable observation has been confirmed and extended by many later investigations.

If the characteristic ring of protein already appears while still adding the nitric acid, then 1 c.c. of the urine in the second measuring glass is diluted with water to 2, 3, 4, etc. c.c., as the case may be, according to the thickness of the ring. In the meanwhile the first measuring glass is emptied and washed, and to it 1 c.c. of the diluted urine is taken and treated with nitric acid as before. If, at this dilution, the protein content is still above 10 mg. per 100 c.c., a further dilution is made in the similar manner on the sample in the second glass until that particular degree of dilution is obtained for which the

1 Skand. Arch. f. Physiol., VI, quoted in Hammarsten's Lehrb. d. Physiol. Chem., 741 (1914).

2 See Pfaundler-Schlossmann's Kinderheilkunde, IV, 24–51 (1910).

time of the appearing of the white ring falls somewhere between ½ and 3 minutes. If the number of milligrams of the protein corresponding to this particular dilution is multiplied by the degree of dilution, the product will give the actual number of milligrams of protein in 100 c.c. original urine. For instance, if the distinct ring appears in 1 minute on a sample diluted to 40 times the original volume, the protein content will be $5 \times 40 = 200$ mg. i.e. the original sample contains 200 mg. protein in 100 c.c.[1]

V. SUMMARY.

(1) The present investigation was conducted with the purpose of finding some rapid methods of approximate estimation of small quantities of protein in solution, esp. in animal fluids such as urine.

(2) The acidimetric estimation of the excess of picric acid after precipitating protein with this acid was found to be feasible in some fluids. The application of this method to the analysis of urine requires a further elaboration.

(3) The precipitato-volumetric estimation of protein in urine by means of picric acid in the medium of one-twentieth Normal acidity of hydrochloric acid has been worked out and proved to be more economical both in time and cost than the Esbach's method in ordinary use among clinicians.

(4) The Roberts-Stolnikow method of estimating minute quantities of protein by the time required for appearance of the characteristic protein ring has been very much simplified and its application extended for the popular family use.

Kyoto, Japan.

1 See for discussion of some confusions frequently arising from some disturbing constituents of urine, Hammarsten's Lehrb. d. Physiol. Chem., 743 (1914).

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